RESEARCH

*Impact of native phosphate solubilizing bacteria on the growth and development of radish (*Raphanus sativus *L.) plants*

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ABSTRACT

This work was aimed at evaluating the activity of native phosphate solubilizing bacteria on growth and development of radish (*Raphanus sativus* L.) plants. Bacteria were isolated from the Departament of Sucre (Colombia) soil using selective media (NBRIP), further identified by macroscopic and microscopic observation and biochemically characterized using the API System. Their ability to solublize phosphate was then evaluated using the vanadomolybdate acid phosphate colorimetric method. The microorganisms of the highest phosphate solubilizing capacity (*Enterobacter* sp. and *Klebsiella* sp.) were multiplied up to concentrations of 10^6, 10^7 and 10^8 c.f.u./mL and then evaluated on radish seeds. Eight treatments were used including witness control and commercial control. Biometric variables, root length, leaf area and dry weight, for treatments T5 (biopreparation with *Klebsiella* sp., 10^7 c.f.u./mL) and T1 (biopreparation with *Enterobacter* sp.,10^6 u.f.c./mL) were statistically higher than the control treatment. In addition, no significant differences regarding dry weight were found with respect to T7 (chemical fertilizer). The growth and development of radish plants were improved in treatments inoculated with bacteria when compared to controls, confirming the positive effect of phosphate solubilizing activity of the native strains.

Keywords: biofertilizers, bioinoculants, phosphate solubilizing microorganisms, biometrics

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RESUMEN

Impact of native phosphate solubilizing bacteria on the growth and development of radish (*Raphanus sativus* L.) plants. El objetivo de este trabajo fue evaluar la actividad solubilizadora de fosfato de algunas bacterias nativas sobre el crecimiento y desarrollo de plantas de rábano (*Raphanus sativus* L.). Las bacterias se aislaron a partir de suelos del Departamento de Sucre (Colombia), usando medio selectivo de crecimiento con fosfato (NBRIP). Posteriormente se identificaron mediante observación macroscópica, microscópica y caracterización bioquímica, empleando el sistema de multipruebas API 20. La propiedad solubilizadora de fosfato se evaluó utilizando el método colorimétrico de ácido vanadomolibdato fosfato. Los microorganismos de más propiedades (*Enterobacter* sp. y *Klebsiella* sp.) se multiplicaron a concentraciones de 10^6, 10^7 y 10^8 u.f.c./mL, y luego se introdujeron en las semillas de rábano. Se utilizaron ocho tratamientos, que incluyeron un control testigo y un control que recibió fertilizante mineral sintético comercial. Las variables biométricas: longitud de la raíz principal, área foliar y peso seco de las plantas que recibieron los tratamientos T5 (biopreparado con *Klebsiella* sp. a 10^7 u.f.c./mL) y T1 (biopreparado con *Enterobacter* sp. a 10^6 u.f.c./mL) fueron estadísticamente superiores al tratamiento control testigo. No hubo diferencias significativas en el peso seco con respecto a las plantas que recibieron T7 (fertilizante mineral sintético comercial). Se evidenció un mayor crecimiento y desarrollo de las plantas de rábano inoculadas con las bacterias, en comparación con los controles, lo que confirmó el efecto positivo de la actividad solubilizadora de fosfato de las cepas nativas.

Palabras clave: biofertilizantes, bioinoculantes, microorganismos solubilizadores de fosfato, parámetros biométricos

*I*ntroduction

Phosphorus is the second most important nutrient for growth and development of both, plants and soil microorganisms [1]. The main physiological functions of this compound in nature deals with micronutrients intake, the increase in biomass of living organisms, metabolic processes of energy transfer, signal transduction, macromolecular biosynthesis, and photosynthesis and respiration chain reactions [2, 3]. Despite being very abundant in the earth as such, it is only found in small proportions in plants so to increase crop production, phosphorus must be provided as a component of chemical fertilizers. However, over 90 % of phosphorus supplemented in chemical fertilizers tends to accumulate in the soil as insoluble compounds that plants cannot use efficiently [4].

The indiscriminate use of chemical fertilizers causes the loss of soil micro and macrobiota together with the accelerated depletion of organic matter and nutrient imbalance, reflected in the loss of fertility and low productivity of soils. Global trends point towards sustainable agriculture, for which should be further encouraged the use and effective treatment of natural resources.

In this regard, bio-fertilizers or microbial inoculants become a vital component of agroecosystems, because they are economically attractive and acceptable

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to reduce the indiscriminate use of chemicals, improving the quantity and quality of domestic remedies [5, 6]. Its use in crops of interest has provided many economic, social and environmental benefits for farmers and producers, due to biofertilizers ability to modify the properties of the soil while keeping its correct nutritional balance. They produce metabolites which facilitate the decomposition of organic material and increase the content of humus. This fact has a positive impact on plant growth and crops quality, as well as improving soil chemical, physical and biological stability [7-9].

Additionally, these bacteria have a key role in maintaining the delicate balance between accumulated and degraded matter. Many of them are efficient in nitrogen fixation or excellent phosphorus solubilization; others produce antioxidants and hormones that stimulate plants growth contributing substantially with farmers to economize chemical fertilizers [10].

The inorganic phosphate solubilizing bacteria have the property of reversing the processes of phosphorus fixation so that, they are related to increase the availability of this element on the soil [11] improving fertility, productivity and crop yields [12]. Several bacterial groups can solubilize phosphates. Among them, the most important genera are *Pseudomonas*, *Bacillus*, *Achromobacter*, *Micrococcus* and *Aerobacter*. These organisms also produce metabolites such as phytohormones or cyanides. Additionally, they can fix nitrogen and synthesize other substances that induce defense against pathogenic organisms or inhibit their growth and development in economically relevant crops [13-15].

The aim of this study was to determine the phosphate solubilizing activity of native bacteria in the growth and development of radish plants (*Raphanus sativus* L.).

Materials and methods

Isolation of phosphate solubilizing strains

The bacteria used were isolated from soil samples from the municipalities of Corozal and San Juan de Betulia (Department of Sucre, Colombia). Samples of 10 g of randomly selected soil squares were dissolved in 90 mL of sterile 1 % NaCl. After 30 min homogenization by stirring, serial dilutions up to $1/10^4$ were done and further plated in Petri dishes containing NBRIP medium (10 g/L glucose, 5 g/L $Ca_3(PO_4)_2$, 5 g/L $MgCl_2 \cdot 6H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L KCl and 0.1 g/L $(NH_4)_2SO_4$) [16] and incubated at 31 °C for 48 h. Colonies forming halos were isolated and replated in NBRIP medium.

Determination of phosphate solubilization activity

The acid colorimetric method of vanadomolybdate phosphate was used to determine the phosphate solubilization activity of each bacterium [17]. The technique was standardized according to the available laboratory equipment conditions, using a standard curve with known concentrations of phosphates. Phosphate solubilizing microorganisms were inoculated on NBRIP liquid medium, and incubated for 48 h, then centrifuged and 7 mL of the supernatant were used for the vanadomolybdate acid phosphate method. Once the absorbance value for each sample was obtained, the phosphate concentrations were calculated by interpolation with the standard curve equation. The procedure for each bacterium was tested in triplicate.

Bacterial Identification and characterization

Colony morphology, size, color, border, elevation and shape of the surface were the used parameters for macroscopic identification of those bacteria with more phosphate solubilizing activity [18]. In microscopic identification, the type of wall and aggregation were considered using specific dyes [19]. The multitests API 20 system (API System SA, La-Balme-les-Grottes, France) and databases apiweb ™ [20] were used to identify the genus and species of the two bacteria.

Preparation of bioinoculants

Bioinoculants were prepared from the selected bacteria able to develop higher phosphate solubilizing activity at concentrations 10^6, 10^7 and 10^8 c.f.u./mL, in NBRIP liquid medium, pH 6.8, with adequate aeration by stirring and agitation for 48 h. The harvested bio-inoculants were used in the following assay.

Phosphate solubilizing activity in radish

Bacterial phosphate solubilizing activity was evaluated in radish seeds (*R. sativus* L.). They were impregnated with bioinoculants in the three concentrations described for 60 min, and then planted in disposable pots containing ground with low phosphorus content (3.19-4.56 ppm).

A completely randomized design (CRD) was used, with eight treatments (T) and 15 replicates each as follows:

T0: control without bioinoculant;

T1, T2 and T3: bioinoculant based on Z_{32} bacterial isolate at 10^6, 10^7 and 10^8 c.f.u./mL, respectively;

T4, T5 and T6: bioinoculant based on Z_{42} bacterial isolate at 10^6, 10^7 and 10^8 c.f.u./mL, respectively;

T7: commercial synthetic mineral fertilizer, containing P_2O_5.

The correct dose to be applied was determined from soil analysis.

Samples were taken at 25 and 50 days after planting (starting test date), and the following biometric parameters were evaluated [21, 22]: a) plant height (cm), from the level of the ground to the furthest leaf of each plant; b) amount of cotyledonal leaves and true photosynthetically active; c) leaf area, measured by the length from the end of the petiole to the tip of the leave and its width, using the ellipse formula to be the closest to the leaves shape; d) primary root length (cm) measured from the base of the stem to the root apex and e) constant dry weight, which was sectioned for each plant roots, stems and leaves, which were dried in a 60 °C oven until a constant dry weight was reached.

The phosphorus content in the analyzed soil was determined [23, 24] and the experiment was conducted with soils displaying the lowest phosphorus content.

Statistical analysis

Data were analyzed with the help Statistix® statistical software version 9.0 (Analytical Software, 2007).

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Data from the CRD were subjected to analysis of variance, which was further assessed by the Tukey's test with a probability of 5 % to determine the best treatment.

Results and discussion

Identification of phosphate solubilizing bacteria

The results or average values of the formed halos diameters and the solubilized phosphate concentration, as consequence of native bacterial isolates action after incubation for nine days at 31 °C, are shown in table 1. Z_{32} and Z_{42} isolates promoted the highest concentrations of solubilized phosphate, reaching values of 596 ppm and 562 ppm, respectively.

The halos sizes in this study were similar to those obtained in other investigations. On NBRIP supplemented medium, diameter halos variations of 4 to 15 mm in size were observed [25], for different bacterial species incubated 11 days at 28 °C. Other studies reported values of 2 mm (*Bacillus polymyxa*), 6 mm (*Pseudomonas aeruginosa*) and 7 mm (*P. fluorescens*) after incubation for 14 days at 28 °C [16].

Bacterial species displaying the best phosphate solubilizing activity were identified. Bacterial isolate Z_{32} corresponded to the genus *Enterobacter* sp. (*Enterobacter cloacae*, with a reliability of 95.1 %, according to the computer program of the database used for this purpose) and the isolated Z_{42} belonged to the genus *Klebsiella* sp. (*Klebsiella oxytoca*, with a reliability of 97.7 %). These microorganisms are gram-negative bacilli. Their colonies are circular, cream-colored, 1-3 mm in diameter, with an entire and elevated edge, and showing a smooth and shiny surface.

Table 1. Halo diameter and phosphate concentration obtained from isolated native phosphate solubilizing bacteria

Bacterial isolates	Halo diameter (mm)	Solubilized phosphate concentration (ppm)
Z_{11}	2	58
Z_{12}	16	232
Z_{13}	0	46
Z_{14}	9	98
Z_{21}	8	108
Z_{31}	3	514
Z_{32}	5	596*
Z_{41}	3	418
Z_{42}	3	562†

* Z_{32}: *Enterobacter cloacae*.
† Z_{42}: *Klebsiella oxytoca*.

The highest values related to zone diameter did not correspond to the highest solubilized phosphate concentrations (Table 1). For example, the bacteria *E. cloacae* (isolate Z_{32}) and *K. oxytoca* (isolate Z_{42}) with smaller diameters halos, displayed the highest phosphate solubilization concentration. Some researchers have found contradictory results between the halo detection method in plates and the phosphate solubilization values in liquid cultures, since many microorganisms do not originate halos on solid media, but can solubilize various types of phosphates in liquid media. Halos formation on solid culture media macroscopically predicts that these microorganisms could solubilize various types of phosphates in liquid media [16, 26].

Evaluation of phosphate solubilizing activity in radish plants

In this research we used the radish (*R. sativus* L.) as a model plant because of its fast growth and genetic homogeneity. Additionally, fruits can be harvested after 30 days of cultivation, and is a plant able to absorb large amounts of phosphorus from the soil [27].

Table 2 displays the averages results of some biometric parameters corresponding to plants height, number of leaves, leaf area, main root length and dry weight.

Plant height

After 25 days of the trial, a better plant growth was evident in treatments T2 (bioinoculant with the *E. cloacae* Z_{32} isolate, 10^6 c.f.u./mL) and T7 (commercial synthetic mineral fertilizer). They had the greatest heights, without significant differences between them. However, in the second evaluation (50 days after planting), growth of T7 plants increased, and their differences became statistically significant compared to the other treatments.

Number of leaves

Regarding the number of leaves, no significant differences were observed after 25 days between the chemical fertilizer and bioinoculants treatments. Nevertheless, there were statistically significant differences

Table 2. Effect of bioinoculants Z_{32} and Z_{42} in growth and development of radish plants (*Raphanus sativus* L.)

Treatments*	Plant height (cm)		Number of leaves		Leaf area (cm^2)		Length of the main root (cm)	Dry weight (g)
	25 days	50 days	25 days	50 days	25 days	50 days	50 days	50 days
T0	7.82^{AB}	11.45^{B}	3.53^{B}	5.66^{A}	26.04^{B}	55.30^{B}	6.97^{B}	0.76^{AB}
T1	7.65^{AB}	11.89^{B}	4.40^{AB}	6.13^{A}	31.78^{AB}	76.35^{B}	7.64^{AB}	1.01^{A}
T2	8.31^{AB}	11.51^{B}	4.66^{AB}	5.20^{A}	37.20^{AB}	76.89^{B}	8.16^{AB}	0.66^{B}
T3	7.59^{AB}	12.06^{B}	4.73^{AB}	5.80^{A}	37.75^{AB}	74.40^{B}	9.35^{AB}	0.78^{AB}
T4	7.20^{AB}	10.82^{B}	4.60^{AB}	5.73^{A}	34.29^{AB}	68.13^{B}	9.01^{AB}	0.66^{B}
T5	6.92^{B}	12.60^{B}	4.53^{AB}	6.00^{A}	32.49^{AB}	76.56^{B}	9.03^{AB}	0.94^{A}
T6	7.39^{AB}	11.12^{B}	4.46^{AB}	5.53^{A}	31.99^{AB}	66.46^{B}	10.48^{A}	0.78^{AB}
T7	9.16^{A}	17.32^{A}	4.86^{A}	6.00^{A}	52.06^{A}	126.15^{A}	9.53^{AB}	1.03^{A}

* Treatments: T0: control without bioinoculant, T1, T2 and T3: bioinoculant Z_{32} (*Enterobacter cloacae*), 10^6, 10^7 and 10^8 c.f.u./mL, respectively, T4, T5, T6: bioinoculant Z_{42} (*Klebsiella oxytoca*), 10^6, 10^7 and 10^8 c.f.u./mL, respectively; T7: commercial synthetic mineral fertilizer containing P_2O_5. Each dose was determined from soil analysis.
$^{A, B}$ Similar letters mean that there are no significant differences with a 5 % probability, according to Tukey's test, from a completely randomized design. The data represent the average of 15 replicates. Statistical comparisons were independent each time for each variable.

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between treatment T7 (commercial chemical fertilizer) and control. In the second evaluation (50 days), all treatments were statistically similar.

Leaf area

At 25 days, although the leaf area of plants treated with fertilizer (T7) was higher, there were no significant differences compared to that of plants treated with bioinoculants. However, after 50 days, the T7 significantly outperformed other treatments. The leaf area of bioinoculant-treated plants was greater than the control. Positive results in radish plants show that inoculation with native microorganisms stimulated the development and growth of the aerial parts of the plants.

This has been reported in other studies pointing out that growth-promoting rhizobacteria as corn plants, with further development of its aerial parts [28, 29]. Moreover, it has been shown that these microorganisms secrete substances that regulate plant growth, since they produce enzymes such as phytase and acid phosphatases, substances that increase soluble phosphorus in soil, stimulate root growth and promote sprouting on different plant species [30, 31].

Taproot length

At 50 days, the T6 (bioinoculant with Z_{42} isolated from *K. oxytoca*, 10^8 c.f.u./mL) stimulated the length of the main root, with statistically significant differences compared to the other treatments, including chemical fertilizer and control. The taproot length of inoculated plants was higher than that of plants under control treatment (Table 1).

Treatments increased the efficiency of nutrient uptake that affects plant growth. These results are probably due to production of native inoculants' phytohormones as auxins and gibberellins, which stimulate the growth, development and enlargement of the root system. Another group [32] described that about 80 % of the isolated strains of *Klebsiella* sp. were also able to fix nitrogen, adhere and colonize the roots, causing an alteration in taproot morphology and increasing the amount of root hairs and the production of bioactive substances (as auxins). Moreover, other reports have demonstrated that the most important plant hormone produced by *Klebsiella* sp. is the auxin indole acetic acid (IAA), which causes morphological changes in the main root. This compound has also been associated with the absorption of minerals, since it promotes a higher biomass production by plants [33-35].

Dry weight

T1 treatment (with bioinoculant Z_{32}, *E. cloacae*, 10^6 c.f.u./mL), T5 (with bioinoculant Z_{42}, *K. oxytoca*, 10^7 c.f.u./mL) and T7 (commercial chemical fertilizer) displayed the best results in regard to radish dry matter. These two treatments were statistically different from the others under study. Bioinoculant treatments T1 and T5 were similar to treatment with mineral fertilizers (T7) in which the benefit of the native phosphate solubilizing bacteria must be highlighted.

The phosphate uptake by plants contributes to increase metabolism, reflected in a higher content of plant organic matter. The assimilation of a great amount of phosphorus positively influences the rapid root formation and growth at seedling stage, accelerates ripening, stimulates fruit coloring and helps seed formation. Besides, it is a component of nucleic acids, phospholipids (essential components of the cell membrane) and the energy transfer molecules such as adenosine triphosphate [36-38].

From the agronomical point of view is very important to notice that plants inoculated with native bacteria were healthy and more vigorous than controls. Interestingly, bacteria of the genera *Enterobacter* sp. and *Klebsiella* sp. are not typical biological control agents. Nevertheless, they can be considered rhizo-competent microorganisms able to form large populations under certain favorable conditions or some preferential substrates. Then, these genera could displace other microorganisms, including pathogens and in this process, reduce the occurrence of disease and influence the healthy growth and development of plants [39].

Coincidently, in a previous study of our group, an *E. cloacae* isolate from the Department of Córdoba, next to Sucre, showed a high phosphate solubilizing ability in vitro [40]. This could led to further studies on the use of bacteria of wider geographic distribution and displaying high phosphate solubilizing activity for scale up as bioinoculants, without the risk of altering the soil native microbiota.

In summary, the results of this study showed that inoculation of radish (*R. sativus* L.) plants with native phosphate solubilizing bacterial isolates of *E. cloacae* (isolate Z_{32}) and *K. oxytoca* (isolate Z_{42}), obtained from soils of the Department of Sucre (Colombia), have beneficial properties for sustainable agriculture with a positive impact on plant growth and development. Bioproducts from the studied strains could be very useful for regional culture inoculation since they may increase the rhizosphere microbial population and contribute to plant nutrition. In turn, the bioinoculants can be an alternative to the use of chemical fertilizers and become in a vital component of sustainable agricultural systems. They are economical and environmentally attractive, would reduce external inputs, improve the quality and production of crops and help to preserve the environment.

The use of beneficial microorganisms as *Enterobacter* sp. and *Klebsiella* sp. can become in a short time, an economical attractive choice to chemical fertilizers on crops of interest, due to their properties to solubilize phosphate deficient soils and improve those deficient in this macronutrient.

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